TITLE OF THE INVENTION

Culture medium for detection of *Dekkera* and *Brettanomyces*

CROSS-REFERENCE TO RELATED APPLICATIONS

Not applicable.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

Not Applicable.

BACKGROUND OF THE INVENTION

The study and characterization of the yeast microflora present in many different habitats (*e.g.* beverages, food, natural substracts) generally involves a first strain isolation and purifying stage in generic yeast culture media, which is followed by a second identification stage by classical taxonomic methods or by molecular biology based techniques. The slow development of the yeasts of the *Dekkera* and *Brettanomyces* genera makes its isolation in the commonly used media extremely difficult, since these yeasts are overtaken by faster developing ones that coexist with them. This fact makes their detection and enumeration, as well as their later identification in food and beverages, rather difficult, this being generally accomplished through the use of very slow, work intensive, and technical skill demanding classical techniques or through molecular biology techniques, involving the use of expensive reactants, molecular probes or primers not always promptly available in the market, and of skilled operators.

It was possible to establish beyond any doubt that these yeasts are involved in the production of a serious organoleptic defect in wines - "horse sweat" - particularly in those that are aged in oak casks. (Chatonnet, *et al.* 1992, *J. Sc. Food Agric.*, 60,165-178). Since then, their detection and enumeration in wines became essential, arising the need for the development of swift methods for that effect. The field bibliography discloses suitable means for the detection and enumeration of yeasts of the *Dekkera* and *Brettanomyces* genera based on this species' resistance to cycloheximide and their acidifying ability (Chatonnet, *et al.* 1992, *J. Sc. Food Agric.*, 60,165-178; Fugelsang, K. *et al.*1993. Ed. Barry H. Gump. ACS Symposium series 536, *American Chemical Society*, Washington. Cap. 7, 110-119; Alguacil, M. *et al.* 1998. *Aliment. Equipos Tecnol*, 10, 81-85). However, the

disclosed media were not entirely satisfactory, since they were not selective enough to prevent the growing of fast developing species, and also were not totally differential.

Therefore, there is a real and effective need for a culture medium and method for the easy and swift identification of yeasts of the *Dekkera* and *Brettanomyces* genera, namely to provide the food and beverage industry a swift method for the isolation, differential detection and enumeration of yeasts of the *Dekkera* and *Brettanomyces* genera.

BRIEF SUMMARY OF THE INVENTION

The present invention refers to a culture medium for the differential detection and enumeration of food and beverage contaminant yeasts of the *Dekkera* and *Brettanomyces* genera, containing ethanol and *p*-cumaric acid in its preparation. It is also an object of the present invention a method using said culture medium for the differential detection and enumeration of yeasts of the *Dekkera* and *Brettanomyces* genera. It is still an object of the present invention the use of said culture medium in a yeast identification gallery.

The objective of the present invention consists in providing the food and beverage industry with a culture medium and a method using said culture medium, able to isolate, differentially detect and number yeasts of the *Dekkera* and *Brettanomyces* genera, by means of the color changing of the culture medium and of the colonies therein developed, and production of a characteristic phenol-like aroma.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING

Not applicable.

DETAILED DESCRIPTION OF THE INVENTION

It was surprisingly found that using a culture medium containing ethanol as the only energy source and *p*-cumaric acid for *Dekkera* and *Brettanomyces* genera yeasts growth, these produce 4-ethylphenol and acetic acid in a characteristic and exclusive form, compared to other yeasts, after incubation for 5 to 12 days. Furthermore, other species of yeasts, usually coexistent with the yeasts of the *Dekkera* and *Brettanomyces* genera, and having a faster development that hinders their detection, are selectively inhibited.

According to the invention, a culture medium was developed, which is partially selective and totally differential for yeasts of the *Dekkera* and *Brettanomyces* genera, which is based on 4-ethylphenol detection, by its characteristic aroma, and acetic acid, by means of an adequate acid-base color change, produced by those yeasts when developed in a ethanol and *p*-cumaric acid containing medium.

Therefore, the present invention refers to a culture medium for the differential detection and enumeration of food and beverage contaminant yeasts of the *Dekkera* and *Brettanomyces* genera, which is composed by a novel combination of compounds with a selective and/or differential effect. Said medium contains ethanol as the sole energy source, that also inhibits other yeast species. It also comprises a nutrient base, ethanol as a non-fermentable energy source and inhibiting other yeasts species, *p*-cumaric acid as an aromatic compound promoting substrate produced by said yeasts species, an acid-base indicator whose turning points lie within the acid range (particularly bromocresol green), an antibiotic that inhibits several species of yeasts (particularly cycloheximide), and agar-agar when the medium is intended for use in a solid form.

In an embodiment, in the culture medium according to the invention the nutrient base is "Yeast Nitrogen Base" and the acid-base indicator is bromocresol green.

In another embodiment, the culture medium according to the invention further contains a bacterial growth inhibitor, particularly chloramphenicol and/or oxytetracycline, which is specially useful for the detection of yeasts of the *Dekkera* and *Brettanomyces* genera within mixed bacteria including populations.

The culture medium according to the invention, when in the liquid form, is prepared by sterilizing filtration, then being dispensed into adequate test tubes. When the medium is desired in the solid form, agar-agar is dissolved in demineralized water and then sterilized in an autoclave; following the sterilization and cooling to about 50°C, the other components are added under aseptic conditions, having previously been filtration sterilized. The pH adjustment for both media is done before the sterilization, to a pH value that depends on the acid-base indicator used. The mixture is homogenized and poured into Petri dishes, the medium being ready for use after solidifying.

The present invention also refers to a method for the detection and enumeration of yeasts of the *Dekkera* and *Brettanomyces* genera, using a partially selective and totally differential culture medium, characterized as above.

The method according to the invention allows the detection and enumeration of yeasts of the *Dekkera* and *Brettanomyces* genera in a simple way using the culture medium according to the present invention.

According to the invention, the culture medium is used by direct contact with a sample under analysis, adequate dilutions being used if the enumeration of the contaminant yeasts is intended. The thus inoculated medium is then incubated at an adequate temperature for the growth of the yeasts, during a time period enough, usually 5 to 10 days, for the development of clearly visible colonies (in case of the solid medium) or for the clouding of the solution (in case of the liquid medium). The detection of the yeasts of the *Dekkera* and *Brettanomyces* genera in the Petri dishes is accomplished by direct observation of cream colored colonies, by the change of medium color according to the type of acid-base indicator that was used, and by the presence of a characteristic phenol-like aroma. If the incubation time is extended, the colonies acquire a darker coloring. The detection in the liquid medium is accomplished by the change of the medium color and by the presence of the phenol-like aroma.

In addition, it is a convenient, swift, and easily reproducible procedure by any microbiological culture media manufacturing laboratory, without the need of the use of new technologies. Once the culture medium is manufactured, its use by any food and beverage industry or quality control laboratory is immediate, since it does not require specialized operators other than the ones in charge of routine microbiological analyses.

Therefore, one of the objectives of the present invention consists in providing the food industry with a procedure that allows the isolation, differential detection and enumeration of yeasts of the *Dekkera* and *Brettanomyces* genera by the production of characteristic colonies, medium color change and production of a phenol-like aroma, thus avoiding the inconveniences of said yeasts identification after their isolation, object being accomplished using the culture medium and method according to the present invention, as described above.

Thus, the use of the medium according to the invention allows:

a) the detection and identification of contaminations in the food and beverage industry due to yeasts of the *Dekkera* and *Brettanomyces* genera, in every step of the manufacturing process, from the raw materials to the finished and stored product.

b) the definition of the critical control points, in order to establish control criteria suited to each one of these points in the food and beverage industry.

Further, the medium according to the invention is useful for inclusion in yeast identification galleries.

Preferred Embodiments of the Invention

In a preferred embodiment, the present invention refers to a culture medium for the differential detection and enumeration of yeasts of the *Dekkera* and *Brettanomyces* genera which comprises "Yeast Nitrogen Base" as a nutrient base in an amount from 5 to 10 g/L, preferably 6.7 g/L, ethanol as a non-fermentable energy source in an amount from 32 to 96 g/L, preferably 48 g/L, *p*-cumaric acid as an aromatic compound (4-ethylphenol) promoting substrate, in an amount from 0.05 to 1.0 g/L, preferably 0.1 g/L, bromocresol green as an acid-base indicator with turning points in the acid range, an antibiotic that inhibits several species of yeasts (cycloheximide) in an amount from 0.004 to 0.1 g/L, preferably 0,01 g/L, and a bacterial growth inhibitor antibiotic.

In this embodiment of the invention, after culture medium inoculation with a sample containing yeasts of the *Dekkera* and *Brettanomyces* genera, which may be a previously isolated sample of these yeasts, or a mixed sample of yeasts and/or yeasts and bacteria, and incubation under advantageous growth conditions for these yeasts genera, after about 5 to 12 days, identification is possible by a culture medium color change, from blue to yellow, development of cream colored colonies and the characteristic phenol-like aroma.

The present invention is further illustrated by means of the following examples, which are intended only to exemplify and by no means limit the scope of the invention.

Examples

Example 1: Preparation of a culture medium according to the invention

The culture medium, object of the present invention, can be prepared using the following formulation : Yeast Nitrogen Base (6.7 g/L), as the nutrient base; ethanol (48 g/L), as the non-fermentable energy source and as an inhibitor for some of the yeasts; *p*-cumaric acid (0.1 g/L), as the phenol-like producing aroma substract; bromocresol green (0.022 g/L), previously dissolved in NaOH, as the acid-base indicator; cycloheximide (0.01 g/L), as the inhibitor antibiotic for some of the yeast species; chloramphenicol (0.1 g/L) and/or oxytetracycline (0.1 g/L), as the bacteria inhibitor antibiotic; and agar-agar (20 g/L), as the gelling agent. The culture medium is sterilized according to the following: the agar-agar is dissolved in 70% of the total needed water, the pH is adjusted to 5.4 with a strong acid, and the resulting solution is sterilized in an autoclave at 120°C, for 20 minutes. The other components are dissolved in the remaining of the demineralized water, the pH is adjusted to 5.4 with a strong acid, and the resulting solution is sterilized by filtration through a 0.22 μm pore diameter membrane. Both of the above solutions are then mixed together when the agar-agar

solution reaches 50°C. The medium is then homogenized and dispensed into Petri dishes, allowing it to solidify prior to the inoculation.

Example 2: Use of the culture medium object of the invention for the detection of yeasts of the *Dekkera* and *Brettanomyces* genera in wines

In this example, two wines suspected of having been altered were analyzed using the culture medium of Example 1. 20 ml samples of each wine were filtered under aseptic conditions, through 0,22 μm pore diameter cellulose acetate membranes. Each membrane was placed on the surface of a Petri dish containing the medium of the invention and incubated at 25°C. After 3 days it was possible to observe colonies in one of the dishes, along with the change of the medium color from blue to yellow; when the dish was opened, the presence of a phenol-like aroma was not detected. In the other dish no colonies were detect after 3 days. After 9 days the dish where the colonies had been observed maintained the same characteristics. In the other dish it was possible to observe small cream colored colonies, a color change of the medium from blue to yellow and a characteristic phenol-like aroma. Using the classical identification methods it was confirmed that the colonies developed in the dish that did not show a phenol-like aroma did not belong to the *Dekkera* or *Brettanomyces* genera, while those of the dish that showed the phenol-like aroma belonged to these genera.

Example 3: *Use of the culture medium object of the invention for the enumeration of yeasts in wines where the presence of filamentous fungi is suspected*

In this case, when the development of molds on the surface of the Petri dishes can shield or inhibit the outcome of yeasts colonies, the Most Probable Number enumeration technique is used, using test tubes with the liquid culture medium. Consequently, agar-agar is not used in the medium formulation, the medium being completely sterilized by filtration. In this instance, the presence of yeasts of the *Dekkera* or *Brettanomyces* genera is detected when there is a clouding of the medium, a turning of its color from blue to yellow, and the presence of a phenol-like aroma; therefore, all the test tubes that show these characteristics are considered positive, and all the others negative.

Although the present invention is described based on its preferred embodiments, it should be apparent to any person skilled in the art that variations and modifications within the spirit and scope of the appended claims are possible.